PB84-184910

Interaction between Normal Human Diploid Cells and Chemical Carcinogens/Mutagens 'In vitro'

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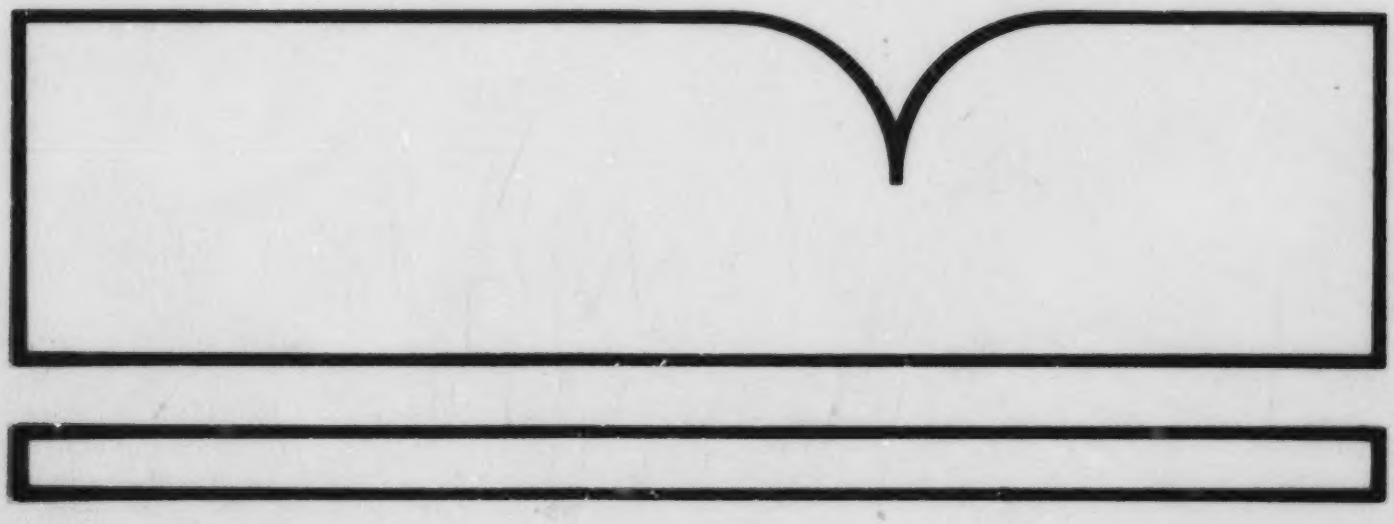
Research Triangle Park, NC

Apr 84



U.S. Department of Commerce
National Technical Information Service
NTIS

PB84-184910

EPA-600/D-84-108
April 1984

INTERACTION BETWEEN NORMAL HUMAN DIPLOID CELLS AND CHEMICAL CARCINOGENS/MUTAGENS IN VITRO

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TECHNICAL REPORT DATA *(Please read Instructions on the reverse before completing)*		
1. REPORT NO. EPA-600/D-84-108	2.	3. RECIPIENT'S ACCESSION NO. PB84 184910
4. TITLE AND SUBTITLE Interaction Between Normal Human Diploid Cells and Chemical Carcinogens/mutagens In Vitro		5. REPORT DATE April 1984
		6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Michael Waters and S. L. Huang*		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Genetic Toxicology Division Health Effects Research Laboratory USEPA Research Triangle Park, NC 27711		10. PROGRAM ELEMENT NO.
		11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Office of Research & Development Health Effects Research Laboratory USEPA Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED
		14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES *Environmental Health Research and Testing, Inc., Research Triangle Park, NC		

16. ABSTRACT

The objectives of the present studies were to develop sensitive, reproducible methods for detecting mutations in normal human fibroblast cells and to demonstrate dose-related mutagenesis by known and potential carcinogens. We have modified conventional test procedures for detecting mutations in human fibroblasts to enhance the systems's sensitivity. These modifications include the use of cell synchronization, multiple treatments and exogenous metabolic activation.

In bacteria and viruses, mutagens act primarily on replicating DNA and preferentially at the replication point. They increase the mutation frequency of a given gene most effectively if administered while that gene is being replicated . Experiments with synchronized mammalian cells have indicated that DNA synthesis is required for mutation.

17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS *(This Report)* Unclassified	21. NO. OF PAGES 19
	20. SECURITY CLASS *(This page)* Unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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INTERACTION BETWEEN NORMAL HUMAN DIPLOID CELLS AND CHEMICAL CARCINOGENS/MUTAGENS IN VITRO

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INTRODUCTION

The objectives of the present studies were to develop sensitive, reproducible methods for detecting mutations in normal human fibroblast cells and to demonstrate dose-related mutagenesis by known and potential carcinogens. We have modified conventional test procedures for detecting mutations in human fibroblasts (Jacobs and DeMars, 1977) to enhance the system's sensitivity. These modifications include the use of cell synchronization (Huang et al., 1981), multiple treatments (Huang et al., 1982), and exogenous metabolic activation (Huang and Waters, 1983).

In bacteria and viruses, mutagens act primarily on replicating DNA and preferentially at the replication point. They increase the mutation frequency of a given gene most effectively if administered while that gene is being replicated (Cerda-Olmedo et al., 1968; Baker and Tessman, 1968; Zampieri et al., 1968; Orkin and Littlefield, 1971; Burke and Fangman, 1975; Kee and Haber, 1975; Suzuki and Okada, 1975). Experiments with synchronized mammalian cells have indicated that DNA synthesis is required for mutation. Mutants resistant to 6-thioguanine (6TG) are induced by 5-bromodeoxyuridine only during early S phase (Aebersold and Burki, 1976; Rasko et al., 1976; Tsutsui et al., 1978). The occurrence of mutations appears to be related to the state of S phase, and the mutation frequencies to the number of S-phase cells in a mutagen-treated cell population. One objective of the present studies was to provide information about conditions for inducing mutations in synchronized cultures of human fibroblasts.

Metabolic activation is necessary to elicit dose-dependent responses in genetic bioassays of most mutagenic and carcinogenic chemicals (Miller, 1970; Heidelberger, 1975). Cell-mediated activation has been used in a few studies with human fibroblasts (Aust et al., 1980; Michalopoulos et al., 1981). Our studies with human fibroblasts have employed rat-liver S-9 microsomal activation (Huang and Waters, 1983). We assayed the activities of 7,12-dimethylbenz[a]anthracene (DMBA) and 4-aminobiphenyl (4AB), with and without S-9 activation, by two different protocols: single treatment of synchronized cells and multiple treatment of nonsynchronized cells. We also assayed the suspected human carcinogens N-ethyl-N-nitrosourea, N,N-bis(2-chloroethyl)-N-nitrosourea, cyclophosphamide, and melphalan in nonsynchronized normal human fibroblasts, using multiple treatments.

In addition, we have used prolonged treatment with MMS to induce mutations in nonsynchronized human fibroblasts, and we have used the Luria-Delbrück fluctuation test (Luria and Delbrück, 1943) to determine the

mutation rate (Huang et al., 1982). The Luria-Delbrück fluctuation test has previously been used to detect the effects of weak mutagens or mutagens at low concentrations (Voogd et al., 1974; Cole et al., 1976; Green et al., 1976).

MATERIALS AND METHODS

Cell Lines, Cultures, Media, and Staining

Human fibroblast lines were derived from neonatal foreskins. Cells were grown in 85 vol of Ham's F10 medium (without hypoxanthine) and 15 vol of fetal bovine serum (FBS) with penicillin (100 IU/ml) and kanomycin (0.1 mg/ml) at 37°C in a 5% carbon dioxide atmosphere. The 6TG selective medium consisted of F10 and FBS with 7.5×10^{-5} M 6TG. Cloning efficiency was measured by inoculating 100 cells into a 60-mm petri dish, renewing the medium after one week, and, at two weeks, rinsing the cells with saline, fixing them with 10% formaldehyde, and staining them with 2% crystal violet.

Metabolic Activation

The S-9 fraction was prepared using 8- to 10-week-old rats (Sprague-Dawley, Madison, WI) primed with Aroclor 1254. Each 10 g of liver was homogenized in 20 ml of 0.25 M sucrose solution and centrifuged at 9000 g. The supernatant fraction (S-9) was collected and stored at -70°C. S-9 batches were prescreened for toxicity; the least toxic batches were used in the experiments. For single treatments, 10% S-9 was used, and for multiple treatments, 0.25% S-9. In methodological control experiments, cell death at these concentration averaged 60%. (The salts and cofactors caused no cell death at the concentrations used; see below.)

Single Treatment of Synchronized Cells (with Metabolic Activation)

Salts and cofactors (23.6 mg of potassium chloride, 22.6 mg of glucose-6-phosphate, 11.6 mg of NADP, and 16 mg of magnesium chloride hexahydrate) were dissolved in 0.4 ml of water. The treatment solution was prepared by mixing 0.4 ml of salts and cofactors, 1 ml of S-9, test chemical dissolved in 0.1 ml of dimethylsulfoxide, and 8.5 ml of F10 medium. Human fibroblasts were synchronized by the method of Milo and DiPaolo (1978). Logarithmically growing cells (approximately 1.5×10^6) were transferred to Dulbecco's modified Eagle's medium (MEM; Biolab, Chicago) lacking arginine and glutamine and supplemented with 10% FBS, 1% nonessential amino acids, and 1% sodium pyruvate. After 24 h, the cells were grown for 16 h in MEM supplemented with 10% FBS plus insulin (0.5 IU/ml). The cells progressed into S phase (Huang et al., 1981) and were exposed to the treatment solution for 4 h in incubators at 37°C in a 5% carbon dioxide atmosphere.

Determination of DNA Synthesis in Synchronized Cell Cultures

For determination of DNA synthesis, [^{3}H]thymidine was added to the above medium to a final concentration of 0.2 μCi/ml At intervals of 1, 2, 3, 4, 5, 6, 7, 8, 16, 17, 18, 20, and 22 h following the release of nutrient stress, cultures received [^{3}H]thymidine and were labelled for 1 h. The cells were then washed twice with saline and dissolved in 0.1 N NaOH. DNA in the

lysate was precipitated with 5% cold tetrachloric acid, and the lysate was filtered through a Whatman GF/C filter paper. The trapped DNA was washed twice with 5% cold tetrachloric acid and then with 95% ethanol. The filter paper was dried and then transferred to a vial containing toluene-based scintillation fluid. The counts for each culture were obtained using a Packard Tri-Carb scintillation counter.

Induction of 6-Thioguanine-Resistance Mutation in Synchronized Cell Cultures During Different Periods of the G_1 and S Phases

Approximately 1.5 x 10^6 cells were plated on 100-mm culture dishes, synchronized, and released from DNA-synthesis blockage. After intervals of 7, 8, 16, 17, 18, 19, 20, and 22 h, the cultures were treated with NA-AAF (4.2 µg/ml) for 1 h.

Mutagenesis with MMS in Cells Encompassing Different Cell Phases

After release from DNA synthesis blockage, the synchronized cells in 100-mm culture dishes were treated with MMS for 16 h. Treatment encompassed the G_1 and S phases (8 to 24 h after release), S and G_2 phases (16 to 32 h after release), and G_2 and G_1 phases (24 to 40 h after release).

Multiple Treatment of Nonsynchronized Cells (with Metabolic Activation)

The chemicals to be tested were dissolved in dimethylsulfoxide or acetone at a concentration of 1 mg/ml and diluted with F10 medium to give the final concentrations (ranging from 0.0015 to 80 µg/ml). Salts and cofactors (147.5 mg of potassium chloride, 141.5 mg of glucose-6-phosphate, 72.5 mg of NADP, and 100 mg of magnesium chloride) and 63 µl of S-9 (or more, depending on its toxicity) were added to each 25 ml of solution. Approximately 2 x 10^5 cells/dish were grown in FBS-F10 medium for 24 h and then exposed to the treatment mixture (5 ml/dish) for 4 h in an incubator at 37°C in 100% air. The cultures were given this 4-h treatment on each of four consecutive days. After each treatment, the cells were rinsed once with F10 medium and then cultured in regular medium.

Prolonged Treatment of Nonsynchronized Cells with MMS

Approximately 1.5 x 10^6 cells were plated on 100-mm dishes for 24 h and then treated daily with MMS in fresh culture medium, for a total treatment time of 24, 48, or 72 h. For longer treatment, a small number of cells were inoculated into culture dishes and exposed to MMS for five days.

Phenotypic Expression and Estimation of Mutation Frequency

After treatment, cells were cultured in FBS-F10 medium for seven days, with two passages. After expression, cells were pooled (five dishes per concentration) for determination of mutation frequencies. Approximately 10^4 cells were plated per 60-mm dish; 120 dishes were prepared for each dose. All dishes received 6TG at a final concentration of 7.5 x 10^{-5} M in 3 h. The 6TG selective medium was renewed once per week. At three weeks, the cells were rinsed with saline, fixed with 10% formaldehyde, and stained with 2% crystal violet. Mutant colonies were counted, and the mutation frequencies determined according to the Poisson expectation (Albertini and

DeMars, 1973; Maher and Wessel, 1975; Jacobs and DeMars, 1977, 1978; Huang and Lieberman, 1978). The mean number of mutant colonies per dish (x) was obtained according to $P_0 = e^{-x}$, where P_0 was the fraction of dishes bearing no mutants. The number of surviving cells per dish was determined by multiplying the initial number of cells per dish (10^4) by the cloning efficiency. Mutation frequency was calculated by dividing the mean number of mutant colonies per dish by the number of surviving cells per dish. Induced mutation frequency was obtained by subtacting the background mutation frequency from the treatment mutation frequency.

Estimation of Mutation Rates Using Luria-Delbrück Fluctuation Analysis

Cells were inoculated in 40 dishes (3000 cells/dish) and allowed to grow in the presence of MMS for five days, with daily renewal of MMS. The number of cells (N_1) was determined for each dish 7 to 9 days after inoculation, and 10^4 cells from each dish were transferred to new dishes. All the new dishes received 6TG at a final concentration of 7.5×10^{-5} M on the same day. The selective medium was renewed once per week. Dishes for estimation of cell cloning efficiency were fixed and stained after two weeks, and those for selection of mutants after three weeks.

Mutation events were assumed to be distributed among the culture dishes randomly (according to a Poisson distribution). The mean number of mutants per dish, x, was estimated as the negative log of P_0. The average number of cell generations per dish, N, was obtained according to the Luria and Delbrück formula $N = (N_1 - N_0)/\ln 2$, where N_0 is the number of cells inoculated before MMS treatment and N_1 is the number of cells at the onset of selection. N_0 and N_1 were corrected for cloning efficiency before mutation rates were calculated. The mutation rate (a) was calculated using the Luria and Delbrück formula $a = x(\ln 2)/(N_1 - N_0)$.

RESULTS

Mutagenic Activities of 7,12-Dimethylbenz(a)anthracene and 4-Aminobiphenyl

Both DMBA and 4AB induced dose-dependent mutations in normal human fibroblasts in the presence of rat S-9 microsomes when administered by either single or multiple treatment (Table 1). The effective chemical concentrations differed between the two methods of administration. Multiple treatment of nonsynchronized cells caused more cell death than did single treatment of synchronized cells, and the effective doses were much lower. Thus, multiple treatment of nonsynchronized cells appeared to be the more sensitive method.

Prolonged Treatment of Nonsynchronized Cultures with MMS

Following repeated treatment with MMS, mutation frequencies in nonsynchronized human fibroblast cultures gradually increased (Table 2). Exposure to MMS for 24 h did not induce mutations in nonsynchronized cultures. However, after a three-day exposure, the increase in mutation frequency was statistically detectable, and a dose-response relationship was demonstrated (Figure 1). The Luria and Delbrück formula was used to calculate mutation rates for cultures exposed to MMS for five days.

TABLE 1. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY 7,12-DIMETHYLBENZ(a)ANTHRACENE AND 4-AMINOBIPHENYL[a]

	Single treatment of synchronized cells			Multiple treatment of nonsynchronized cells		
Conc. (μg/ml)	Relative cloning effic. (%)	Mean mutants per dish	Induced mutation freq. (x 10^{-6})	Relative cloning effic. (%)	Mean mutants per dish	Induced mutation freq. (x 10^{-6})
7,12-DIMETHYLBENZ(a)ANTHRACENE						
0	100	0.009	--	100	0.124	--
0.20				108	0.192	21.30
0.39				94	0.288	73.50**
0.78				105	0.341	79.80**
1.56				89	0.325	97.30**
3.13	95	0.017	2.47	61	0.452	248.80**
6.25	90	0.027	5.68			
12.50	90	0.073	19.43*			
25.00	80	0.077	23.58**			
50.00	63	0.146	59.85**			
4-AMINOBIPHENYL						
0	100	0.008	--	100	0	--
3.13				85	0.025	11.5
6.25				99	0.051	20.1*
12.50				98	0.087	34.4*
25.00				84	0.118	54.5**
40.00	95	0.033	4.41			
60.00	65	0.042	15.54*			
80.00	80	0.060	18.58*			

[a]Linear regressions: DMBA: single treatment, $\ln(y) = -0.27 + 1.13 \ln(x)$
multiple treatment, $\ln(y) = 4.55 + 0.75 \ln(x)$
4AB: single treatment, $\ln(y) = -6.34 + 2.15 \ln(x)$
multiple treatment, $\ln(y) = 1.61 + 0.75 \ln(x)$

*Significantly different from control, $p < 0.05$.
**Significantly different from control, $p < 0.01$.

TABLE 2. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY REPEATED TREATMENT WITH MMS

Treatment period (h)	Conc. (μg/ml)	Cloning effic. (%)	P_0	No. of mutants per dish	No. of viable cells per dish	Mutation freq. (x 10^{-6})	Induced mutation (x 10^{-6})
24	0	33.4	0.962	0.038	3674	10.47	--
	3	34.6	0.904	0.101	3806	26.56	16.09
	6	34.4	0.918	0.085	3784	22.50	12.03
	12	37.6	0.892	0.114	4136	27.59	17.12
48	0	33.8	0.973	0.028	3718	8.18	--
	3	34.0	0.900	0.105	3740	30.98	22.80
	6	35.2	0.909	0.095	3872	27.08	18.90
	12	38.8	0.836	0.179	4268	46.05	37.87
72	0	33.8	0.973	0.028	3718	8.18	--
	3	28.8	0.927	0.076	3168	26.22	18.04
	6	27.8	0.911	0.094	3058	33.64	25.46
	12	31.6	0.862	0.148	3476	46.85	38.67

Spontaneous mutation rates were estimated to be 4 to 20 x 10^{-7}/cell per generation. Mutation rates were significantly higher in treated cultures than in controls, and induced mutation rates increased linearly with increasing concentrations of MMS (Figure 2). Prolonged treatment increased the fraction of dishes with mutants.

Multiple Treatment of Nonsynchronized Cultures with Four Suspected Human Carcinogens

All four agents caused dose-dependent mutations when administered in multiple treatments to nonsynchronized human fibroblast cultures (Tables 3 and 4). N-ethyl-N-nitrosourea was found to be a potent direct-acting mutagen, and N,N-bis(2-chloroethyl)-N-nitrosourea to be a weaker direct-acting mutagen. Both melphalan and cyclophosphamide required metabolic activation, and melphalan appeared to be the more potent mutagen. Neither melphalan nor cyclophosphamide caused dose-dependent mutation when administered to synchronized cells in a single treatment. The results obtained with the multiple treatment method confirm the mutagenicity observed with these agents in other genetic bioassays. Multiple treatment of nonsynchronized human fibroblast cultures appears to be a relatively sensitive method for testing mutagenicity in human cells.

Cell Synchronization

Dulbecco's MEM lacking arginine and glutamine and supplemented with 10% FBS, 1% nonessential amino acids, and 1% sodium pyruvate stopped most cell growth and decreased DNA synthesis; the cells were blocked in G_1/S phase within 24 h. However, as shown in Figure 3, some cells escaped blockage and

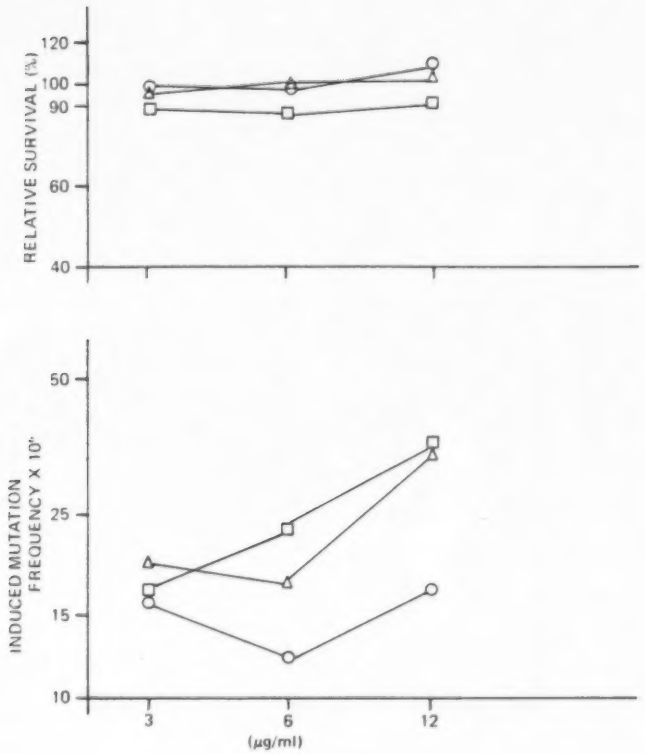

Figure 1. Mutagenesis and cytotoxicity in nonsynchronized human fibroblasts treated with MMS for 24 h (O), 48 h (Δ), or 72 h (□). The linear regression for mutation frequency after 72-h treatment is $\ln(y) = 2.29 + 0.55 \ln(x)$.

continued DNA synthesis. MEM supplemented with 10% FCS plus insulin (0.5 IU/ml) released cells from G_1/S interphase blockage. New DNA synthesis began at 16 h after release and peaked at 20 h. As shown in Figure 3, the increase in DNA synthesis did not result from an increase in numbers of cells.

One-hour exposure of cells to NA-AAF at different stages of the cell cycle allowed observation of the temporal pattern of mutation. Exposure of cells during the G_1 phase produced no obvious mutations (Table 5); mutations were induced only during S phase. Mutations frequencies increased as S phase progressed, were highest when DNA synthesis reached a maximum, and declined after the midpoint of S phase, even though DNA synthesis continued in some portion of the cell population. MMS treatment starting during G_1 and including the entire S phase induced mutations in a dose-dependent fashion, as did treatment including S phase and G_2 phase (Table 6). However, treatment including G_2 and G_1 phases did not induce mutations. Induction of 6TG resistance was linear with increasing dose, and mutation induction

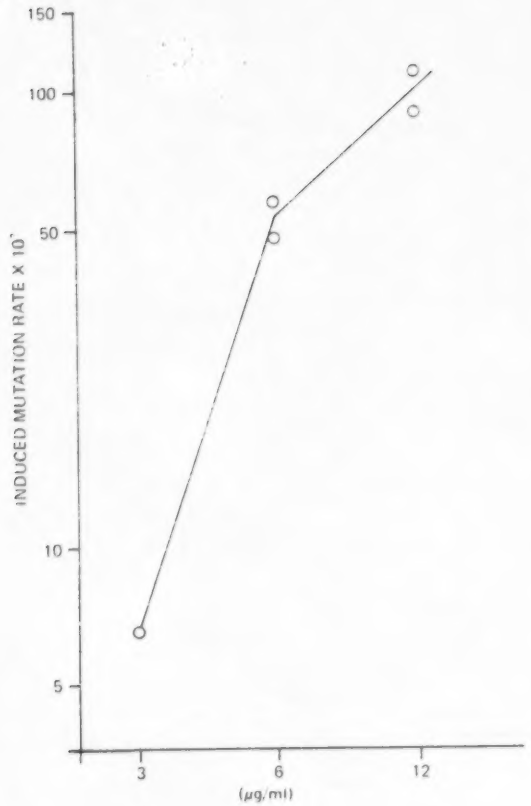

Figure 2. Mutation rates for human fibroblasts treated with MMS for five consecutive days, calculated by the Luria and Delbrück formula. The linear regression for mutation frequency after five-day treatment is $\ln(y) = 0.11 + 1.91 \ln(x)$.

followed one-hit kinetics. These findings indicate that incorporation of mutagens during DNA synthesis was required for mutation.

DISCUSSION

Synchronized human fibroblast cultures were obtained by the amino acid deprivation method of Milo and DiPaolo (1978). New DNA synthesis began 16 h after release from nutrient stress and peaked at 20 h. This permitted division of S phase into eight 1-h periods. The cellular effects of direct perturbation of DNA during various portions of the DNA synthetic period were examined. Mutation frequency was found to depend on the portion of S phase during which treatment was administered. Similar results have been reported for the HGPRT locus in Chinese hamster cells (Aebersold and Burki, 1976; Rasko et al., 1976) and Syrian hamster cells (Tsutsui et al., 1978). Treatment of S-phase human fibroblasts with MMS, NA-AAF, and MNNG induces

TABLE 3. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY REPEATED TREATMENT WITH N-ETHYL-N-NITROSOUREA AND N,N-BIS(2-CHLOROETHYL)-N-NITROSOUREA

Conc. (μg/ml)	Relative cloning effic. (%)	P_0	No. of mutants per dish	No. of viable cells per dish	Mutation freq. (x 10^{-6})	Induced mutation (x 10^{-6})
N-ETHYL-N-NITROSOUREA						
0	100	0	0	2866	0	0
0.78	97	0.975	0.025	2766	9.04	9.04
1.56	95	0.975	0.025	2713	9.22	9.22
3.13	91	0.950	0.051	2600	19.62	19.62
6.25	89	0.831	0.186	2547	73.03	73.03
12.50	91	0.808	0.213	2613	81.52	81.52
25.0	73	0.658	0.418	2100	199.05	199.05
N,N-BIS(2-CHLOROETHYL)-N-NITROSOUREA						
Experiment 1						
0	100	0.941	0.061	2373	25.71	0
0.006	89	0.928	0.075	2113	35.50	9.79
0.012	97	0.890	0.117	2313	50.58	24.87
0.024	94	0.858	0.153	2220	68.92	43.21
0.048	83	0.882	0.126	1980	63.64	37.93
Experiment 2						
0	100	0.983	0.017	3690	4.60	0
0.006	100	0.967	0.034	3680	9.20	4.6
0.012	81	0.922	0.081	2980	27.2	22.6
0.024	72	0.894	0.112	2670	41.9	37.3
0.048	78	0.928	0.075	2888	26.0	21.4

mutation (Huang et al., 1981). The present report and others show that only S-phase cells are sensitive to mutagens and that S-phase cells must be included in mutagenesis experiments (Taylor, 1960; Bader et al., 1963; Kikuchi and Sandberg, 1964; Mueller and Kajiwara, 1966; Mukherjee et al., 1968; Braun and Willis, 1969). Mutation can be induced in logarithmically growing human cells (Albertini and DeMars, 1973; Cox and Masson, 1974; Van Zeeland et al., 1974; Kuroda, 1975; Maher and Wessel, 1975; Jacobs and DeMars, 1978; Huang and Lieberman, 1978). Such cultures contain various proportions of S-phase cells. Cell culture synchrony was used here to accumulate larger numbers of S-phase cells for mutagen treatment and thus to increase the sensitivity of the assay.

TABLE 4. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY REPEATED TREATMENT WITH CYCLOPHOSPHAMIDE AND MELPHALAN

Conc. (μg/ml)	Relative cloning effic. (%)	P_0	No. of mutants per dish	No. of viable cells per dish	Mutation freq. (x 10^{-6})	Induced mutation (x 10^{-6})
CYCLOPHOSPHAMIDE						
Without Activation						
0	100	0.958	0.043	2293	18.8	--
0.014	105	0.992	0.008	2407	3.3	--
0.028	114	0.955	0.046	2620	17.6	--
0.056	103	0.992	0.008	2353	3.4	--
0.110	102	0.983	0.017	2340	7.3	--
0.220	116	1.000	0	2667	0	--
0.440	91	0.983	0.017	2080	8.2	--
With S-9 Activation						
0	100	0.983	0.017	2760	6.2	0
0.014	91	0.975	0.025	2500	10.0	3.8
0.028	100	0.974	0.026	2760	9.4	3.2
0.056	89	0.967	0.034	2460	13.8	7.6
0.110	82	0.967	0.034	2267	15.0	8.8
0.220	93	0.926	0.077	2573	29.9	23.7
0.440	83	0.918	0.086	2290	37.6	31.4
MELPHALAN						
Without Activation						
0	100	0.854	0.157	4030	39.1	--
0.00078	99	0.900	0.105	3980	26.4	--
0.00156	102	0.912	0.092	4110	22.4	--
0.00313	100	0.890	0.162	4030	40.2	1.1
0.00625	101	0.851	0.161	4080	39.5	0.4
0.01250	99	0.866	0.144	3990	36.1	--
With S-9 Activation						
0	100	0.954	0.047	2360	19.9	--
0.00078	89	0.951	0.050	2093	23.9	4.0
0.00156	78	0.925	0.078	1852	42.1	22.2
0.00313	82	0.914	0.090	1933	46.6	26.7
0.00625	83	0.908	0.097	1955	49.6	29.7
0.01250	81	0.838	0.176	1920	91.7	71.8

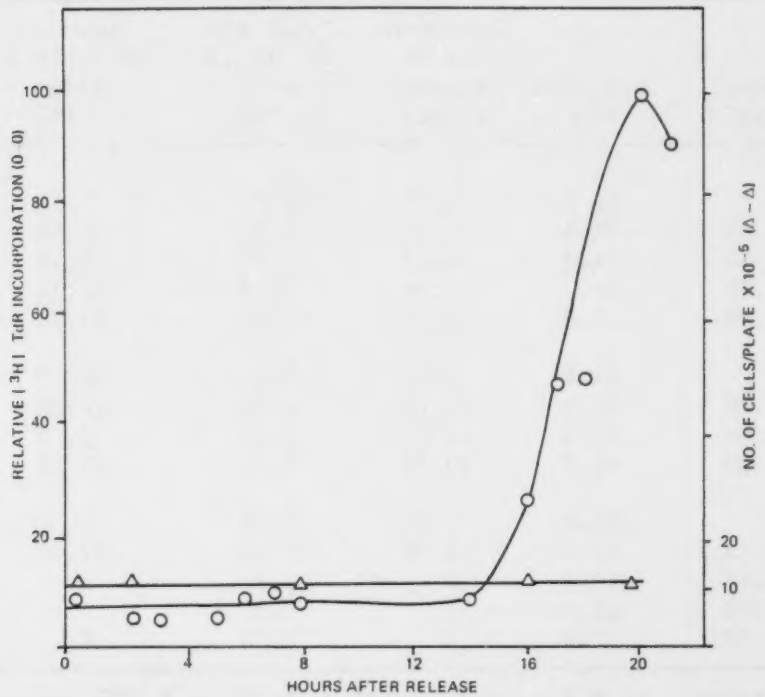

Figure 3. Relative DNA synthesis by human fibroblasts at different phases of the cell cycle after release from blockage.

TABLE 5. INDUCTION OF MUTATIONS IN HUMAN FIBROBLASTS BY NA-AAF DURING DIFFERENT PHASES OF THE CELL CYCLE

Hours after release	cpm	Number of cells/dish (x 10^5)	Mutation frequency (x 10^{-6})	
			Control	Treated
7	263	1.31	3.9	0
8	215			0
16	804	1.44	11	29
17	1427	1.39		
18	1448			96
20	3065	1.39		127
22	2600		10	76

TABLE 6. INDUCTION OF MUTATIONS IN HUMAN FIBROBLASTS BY MMS DURING DIFFERENT PHASES OF THE CELL CYCLE[a]

Cell phase	Conc. (μg/ml)	Survival (%)	Corrected no. of mutant clones	Total no. of viable cells (x 10^5)	Mutants per viable cells (x 10^{-6})	Induced mutation freqency (x 10^{-6})
G_1 + S	0	32.4	1.91	2.66	7.18	
	6.25	32.6	3.52	2.97	11.86	4.68
	12.50	28.8	6.47	2.53	25.58	18.40
	25.00	28.2	14.66	2.43	60.34	53.16
	50.00	22.8	16.18	1.98	81.72	74.54
S + G_2	0	46.8	6.73	3.70	18.19	
	6.25	47.0	15.74	3.85	40.88	21.81
	12.50	47.4	23.62	3.93	60.10	41.91
	25.00	44.0	40.55	3.83	105.87	87.68
G_2 + G_1	0	21.6	5.08	2.38	21.36	
	3.13	19.0	5.74	2.09	27.44	
	6.25	20.2	0	2.22	0	
	12.50	12.4	0	1.36	0	
	25.00	27.6	0	3.04	0	

[a]Linear regression: G_1 + S phases, $\ln(y) = -0.653 + 1.339 \ln(x)$
S + G_2 phases, $\ln(y) = -0.49 + 0.98 \ln(x)$

Our choice of methods for the present studies (single treatment of synchronized cells and multiple treatment of nonsynchronized cells) reflects previous experience with these and other combinations of culture growth state and treatment regimen. With synchronized cell cultures, our previous studies comparing single and multiple treatment have led us to discontinue work with the latter treatment regimen. The differential effects of chemical and/or concentration on cell cycling make it very difficult to predict, following multiple treatment, the time at which a synchronized culture will pass into S phase. With nonsynchronized cultures, we have observed that known mutagens are more likely to cause dose-dependent mutations when administered by multiple treatment. For example, three or more treatments with MMS are needed to elicit dose-dependent mutations in nonsynchronized cells (Huang et al., 1982). MMS is considered a weak mutagen and, in that respect, is representative of most environmental mutagens of interest. Thus, our work with nonsynchronized cells has increasingly emphasized multiple treatment.

As shown in Table 1, the effective chemical concentrations of DMBA and 4AB differed markedly between single treatment of synchronized cultures and multiple treatment of nonsynchronized cultures. Multiple treatment caused more cell death, and the effective doses were much lower. Because more

mutations were induced at lower concentrations, multiple treatment of nonsynchronized cells appears to be the more sensitive method. Results obtained with N-ethyl-N-nitrosourea, N,N-bis(2-chloroethyl)-N-nitrosourea, melphalan, and cyclophosphamide (Tables 3 and 4) tend to confirm that multiple treatment of nonsynchronized cells is the more sensitive of the two methods reported here. All four chemicals caused dose-dependent mutation when administered to nonsynchronized cells by multiple treatment, but two (melphalan and cyclophosphamide) failed to do so when administered to synchronized cells in a single treatment (Huang, unpublished data).

MMS induces 6TG resistance in CHO cells (Couch et al., 1978), mutations at the TK locus in mouse lymphoma cells (Cole and Arlett, 1978) and sister-chromatid exchanges and chromosomal aberrations in cultured cells (Palitti and Becchetti, 1977; Raff and Sugahara, 1980). MMS does not induce Oua-resistant variants in mouse lymphoma cells when tested by conventional methods (Cole and Arlett, 1978). However, the ability of MMS to induce Oua-resistance in mouse lymphoma cells was demonstrated through the use of the fluctuation test (Cole et al., 1976). This result was attributed to the effects of prolonged treatment. Similarly, in our experiments, MMS did not induce mutation in human fibroblasts when tested by conventional methods (Jacobs and DeMars, 1977), but prolonged treatment with MMS produced significant dose-dependent mutation, as did the use of the Luria and Delbrück experimental design. Using the conventional protocol, we were also able to demonstrate mutagenicity of MMS in synchronized human fibroblast cultures (Huang et al., 1981). These results are explained by the necessity of including S phase cells in mutagenicity assay. Both cell synchronization and prolonged treatment increase the sensitivity of the test system, by increasing the opportunity for S-phase cells to be exposed to the mutagen, thus increasing the number of mutants per culture.

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